β-AMYLOID PEPTIDE-BINDING PROTEINS AND POLYNUCLEOTIDES ENCODING THE SAME

This application claims benefit of U.S. Provisional Application 60/064,583, filed April 16, 1997, the content of which is incorporated into this application by reference.

Field of the Invention

The present invention relates to a novel polynucleotides and proteins encoded by such polynucleotides, along with therapeutic, diagnostic, and research utilities for these polynucleotides and proteins. In particular, the invention relates to polynucleotides and proteins encoded by such polynucleotides which bind to b-amyloid peptide, one of the primary components of amyloid deposits associated with Alzheimer's Disease.

Background of the Invention

Alzheimer's disease (AD) is a progressive dementing disorder of the elderly characterized by a series of structural abnormalities of the brain. Neurons in multiple regions of the central nervous system (CNS) become dysfunctional and die, resulting in alterations in synaptic inputs. Cell bodies and proximal dendrites of these vulnerable neurons contain neurofibrillary tangles composed of paired helical filaments, the main component of which is a phosphorylated microtubular-binding protein, namely tau. One of the hallmarks of the disease is the accumulation of amyloid containing deposits within the brain called senile (or neuritic) plaques. The principal component of amyloid plaques is b-amyloid peptide (hereinafter "BAP", also referred in the literature as Aβ, βAP, etc.) which forms dense aggregates during the course of AD.

BAP is a 39-43 amino acid peptide derived by proteolytic cleavage of amyloid precursor protein (hereinafter "APP") and composed of a portion of the transmembrane domain and the luminal/extracellular domain of APP. It is thought that the BAP peptide comprising 42 amino acids

(BAP42) is potentially the more toxic aggregated form in humans. APP occurs as several BAP-containing isoforms. The major forms are comprised of 695, 751, and 770 amino acids, with the latter two APP containing a domain that shares structural and functional homologies with Kunitz serine protease inhibitors. In normal individuals, BAP does not accumulate and is rapidly removed from circulating fluids. However, the peptide can form plaques on surfaces of dystrophic dendrites and axons, microglia, and reactive astrocytes. The aggregation and deposition of BAP in neuritic plaques is postulated as one of the initiating events of AD. Investigation of the events leading to the expression and consequences of BAP and their individual roles in AD is a major focus of neuroscience research. In particular, the discovery of proteins that bind BAP is critical to advance understanding of the pathogenesis of the disease and to potentially introduce novel therapeutic targets.

Until the present invention, proteins and fragments thereof which bind with human BAP and which may be involved in the biological effects of BAP in AD had not been identified.

Summary of the Invention

This invention provides novel isolated polynucleotides which encode gene products that selectively bind human β-amyloid peptide (BAP) amino acid sequences.

In one embodiment, the present invention provides a composition comprising an isolated polynucleotide selected from the group consisting of:

(a) polynucleotide comprising the nucleotide sequence of SEQ ID NO: 1;

(b) a polynucleotide comprising the nucleotide sequence of a β-amyloid peptide-binding protein (BBP) of clone BBP1-fl deposited under accession number ATCC 98617;

(c) a polynucleotide encoding a β-amyloid peptide-binding protein (BBP) encoded by the cDNA insert of clone BBP1-fl deposited under accession number ATCC 98617;

(d) a polynucleotide comprising the nucleotide sequence of SEQ ID NO: 1 from nucleotide 202 to nucleotide 807;

(e) a polynucleotide comprising the nucleotide sequence of a β-amyloid peptide-binding protein (BBP) of clone pEK196 deposited under accession number ATCC 98399;

(f) a polynucleotide encoding a β-amyloid peptide-binding protein (BBP) encoded by the cDNA insert of clone pEK196 deposited under accession number ATCC 98399;

(g) a polynucleotide encoding a protein comprising the amino acid sequence of SEQ ID NO: 2;

(h) a polynucleotide encoding a protein comprising a fragment of the amino acid sequence of SEQ ID NO: 2 having human β-amyloid peptide binding activity, the fragment comprising the amino acid sequence from amino acid 68 to amino acid 269 of SEQ ID NO: 2;

(j) a polynucleotide which is an allelic variant of the polynucleotide of (a)-(f) above;

(k) a polynucleotide which encodes a species homologue of the protein of (g)-(i) above; and

(l) a polynucleotide capable of hybridizing under stringent conditions to any one of the polynucleotides specified in (a)-(h).

Preferably such polynucleotide comprises the nucleotide sequence of SEQ ID NO: 1; the nucleotide sequence of a β-amyloid peptide-binding protein (BBP) of clone BBP1-fl deposited under accession number ATCC 98617; or a polynucleotide encoding a β-amyloid peptide-binding protein (BBP) encoded by the cDNA insert of clone BBP1-fl deposited under

accession number ATCC 98617. Another embodiment provides the gene corresponding to the cDNA sequence of SEQ ID NO: 1.

In other embodiments, the present invention provides a composition comprising a protein, wherein said protein comprises an amino acid sequence selected from the group consisting of:

(a) the amino acid sequence of SEQ ID NO: 2;

(b) the amino acid sequence of SEQ ID NO: 2 from amino acid 68 to amino acid 269;

(c) the amino acid sequence encoded by the cDNA insert of clone BBP1-fl deposited under accession number ATCC 98617; and

(d) fragments of the amino acid sequence of SEQ ID NO: 2 comprising the amino acid sequence from amino acid 185 to amino acid 217 of SEQ ID NO: 2.

Preferably such protein comprises the amino acid sequence of SEQ ID NO: 2 or the amino acid sequence of SEQ ID NO: 2 from amino acid 68 to amino acid 269. Fusion proteins are also claimed in the present invention.

In certain preferred embodiments, the polynucleotide is operably linked to an expression control sequence. The invention also provides a host cell, including bacterial, yeast, insect, and mammalian cells, transformed with such polynucleotides compositions.

Processes are also provided for producing a BBP which comprises (a) growing a culture of the host cell of claim 3 in a suitable culture medium; and (b) purifying the protein from the culture medium.

Compositions comprising an antibody which specifically reacts with such BBPs are also provided by the present invention.

Methods and diagnostic processes are provided for detecting a disease state characterized by the aberrant expression of human BAP, as well as methods for identifying compounds which regulate the activity of BBPs.

Another embodiment of the invention includes transgenic animals comprising a polynucleotide encoding a BBP operably linked to an expression control sequence.

Brief Description of the Drawings

The following drawings depict certain embodiments of the invention. They are illustrative only and do not limit the invention otherwise disclosed herein.

Figure 1: Yeast 2-hybrid screen design. A Y2H host strain expressing the Gal4 DNA-binding domain fused to BAP_{42} (BAP^{BD}; plasmid containing TRP1 marker) and nonfusion BAP_{42} (BAP; plasmid containing URA3 marker) was transformed with a Y2H human fetal brain cDNA library (plasmid containing LEU2 marker) expressing Gal4 activation domain fusion proteins ($unknown^{AD}$) as described. Therefore, strains contained three episomal plasmids, denoted by circles, expressing the indicated protein. Positive protein-protein interactions reconstituted Gal4 activity at the upstream activating sequence (GALUAS) thereby inducing transcription of the reporter gene HIS3.

Figure 2: Demonstration of BBP1/BAP association. Y2H strains were assayed for histidine prototrophy by making 10-fold serial dilutions and spotting 5 μl on synthetic agar medium lacking tryptophan, leucine, histidine and containing 25 mM 3-amino-triazole as described. All strains contain the BAP fusion protein expression plasmid pEK162 as indicated by the label BAP. The first columns (vector) contain independently derived strains carrying pEK162 and the vector pACT2 expressing an irrelevant fusion protein. These serve as a measure of background for comparison with strains expressing target proteins. The columns marked by BBP1Dtm express a truncated BBP1 from pEK198, as described in the text. The interaction between BAP and BBP1Dtm fusion proteins reconstitutes Gal4 activity, resulting in induction of a HIS3 reporter gene (see Figure 1), observed as enhanced prototrophic growth compared to the control strains.

Figure 3: Bioassays demonstrating BBP1 interactions with Gα proteins. The predicted intracellular domain of BBP1 was expressed as a Gal4 DNA-binding domain with portions of rat Gas, Gao, or Gai2 expressed as Gal4 activation domain fusion proteins. Y2H responses of two independently derived clones of each strain were compared to responses of cells lacking a G protein component (vector). The protocol is as described in the legend to Figure 2.

Figure 4: Localization of the interactions between BBP1 and BAP. BBP1Δtm was divided into two overlapping segments as described in the text. These proteins, BBP1ΔC or BBP1ΔN, were assayed for interactions with BAP. The assay method and the strains labeled vector or BBP1Δtm are as described in the legend to Figure 2. Strains labeled BBP1ΔC or BBP1ΔN express the indicated BBP1 segment as a fusion protein.

Figure 5: Expression of BBP1 mRNA in human tissues (A) and brain regions (B). Nylon membranes blotted with 2 μg size fractionated poly-A RNA isolated from the indicated tissues were obtained from CLONTECH. These were hybridized with a radiolabeled BBP1 cDNA probe as described. A predominant band corresponding to 1.25 kb (determined from molecular weight markers, not shown) was observed in all lanes. Higher molecular weight bands likely correspond to heteronuclear RNA; the BBP1 gene contains several introns. Blots were stripped and reprobed with β-actin as a loading and RNA integrity control; all lanes exhibited equivalent signal (data not shown).

Figure 6: Expression of BBP1 and APP in cells of the hippocampus. Images of in situ hybridization autoradiograms showing the pattern of BBP1 (A) and APP (B) expression in human hippocampal and entorhinal cortex. The sections used to generate these images were taken from postmortem specimens obtained from two different patients. Abbreviations: DG = dentate gyrus; CA1 = hippocampal subfield; EC = entorhinal cortex.

Figure 7: Comparison of BBP1 interactions with human or rodent BAP. Rodent BAP was engineered and expressed as a fusion protein as described in the text. The strains labeled human BAP are identical to those shown in Figure 2. The strains labeled rodent BAP express rodent BAP as the Gal4 DNA-binding domain fusion. Vector indicates control strains containing only vector opposing the BAP fusion proteins; BBP1 indicates strains expressing the BBP1Δtm fusion protein.

Detailed Description of the invention

The present invention relates to the isolation and cloning of a human β-amyloid peptide binding protein (BBP1). BBP1 has been characterized as a fusion protein in a yeast 2 hybrid assay as binding to the 42 amino acid fragment of BAP (BAP42). Expression of BBP1 has been shown in human tissues and in specific brain regions (Figure 5). Importantly BBP1 has been demonstrated to selectively bind human BAP in a yeast 2 hybrid system as compared to rodent BAP. These findings support the premise that the BBP1 of the present invention may be used in the diagnosis and treatment of Alzheimer's Disease as well as to evaluate and screen drugs to regulate the accumulation of amyloid-containing plaques in the brain.

The BBP1 Coding Sequence

The initial human BBP1 clone (designated clone 14) was obtained by using a yeast 2-hybrid (Y2H) genetic screen developed to identify proteins which interact with human BAP_{42}, a potentially more toxic form of BAP. BAP_{42} was expressed fused to the yeast Gal4 DNA-binding domain and was also expressed as free peptide (Figure 1). This strain was transformed with a human fetal brain cDNA Y2H library. A single clone, denoted #14, from approximately 10^6 independent transformants, produced consistent reporter gene activation and contained a substantial open reading frame continuous with that of the GAL4 domain. The cDNA insert comprised 984 base pairs, terminating in a poly-A tract. This sequence encoded 201 amino acids (amino acid 68 to amino acid 269 of

SEQ ID NO: 2) with two regions of sufficient length and hydrophobicity to transverse a cellular membrane. There are also potential asparagine-linked glycosylation sites. Clone 14 was designated clone pEK196 and deposited as ATCC 98399 .

The library-derived plasmid was isolated from clone 14 and used to reconstruct Y2H assay strains. Examination of these strains demonstrated that the BAP fusion protein specifically interacted with the clone 14 protein, although the response was weak. Since protein domains of strong hydrophobicity, such as transmembrane regions, inhibit Y2H responses (Ozenberger, unpublished data), clone 14 insert was truncated (BBP1Δtm; see Table 2 below for further description) to remove the region of strongest hydrophobicity and retested for interactions with BAP. A much more robust Y2H response was observed with BBP1Δtm, supporting the notion that the deleted sequences encode a potential transmembrane ("tm") anchor. Clone 14 identifies a novel BAP binding protein in the form of a fusion protein.

The BBP1 cDNA sequences contained in clone 14 were identified as lacking the 5' end of the protein coding region as no potential initiating methionine codon was present. Multiple attempts at conventional 5' RACE (rapid amplification of cDNA ends) utilizing a standard reverse-transcriptase only resulted in the addition of 27 nucleotides. Thus, a genomic cloning approach as described in Example 2, below, was used to isolate the 5' terminus.

Since the 5' coding sequence terminus was derived from a genomic library, there existed the possibility that this region contained introns. This potentiality was investigated by two methods as described in Example 2, below. The resulting data confirmed the upstream sequences (both from genomic and cDNA sources) and the lack of introns in this region. Plasmid BBP1-fl containing a cDNA insert encoding the full length BBP1 protein coding region was deposited in the American Type Culture Collection with accession number 98617. The entire coding

region and deduced protein sequence is shown in SEQ ID NOS:1 and 2..The 3' nontranslated nucleotide sequences are contained in the original clone 14 (pEK196).

In accordance with the present invention, nucleotide sequences which encode BBP1, fragments, fusion proteins or functional equivalents thereof, may be used to generate recombinant DNA molecules that direct the expression of BBP1, or a functionally active peptide, in appropriate host cells. Alternatively, nucleotide sequences which hybridize to portions of the BBP1 sequence may be used in nucleic acid hybridization assays, Southern and Northern blot assays, etc.

The invention also includes polynucleotides with sequences complementary to those of the polynucleotides disclosed herein.

The present invention also includes polynucleotides capable of hybridizing under reduced stringency conditions, more preferably stringent conditions, and most preferably highly stringent conditions, to polynucleotides described herein. Examples of stringency conditions are shown in the table below: highly stringent conditions are those that are at least as stringent as, for example, conditions A-F; stringent conditions are at least as stringent as, for example, conditions G-L; and reduced stringency conditions are at least as stringent as, for example, conditions M-R.

Stringency Conditions

Stringency Condition	Polynucleotide Hybrid	Hybrid Length (bp)[1]	Hybridization Temperature and BufferH	Wash Temperature and BufferH
A	DNA:DNA	$ 50	65EC; 1xSSC -or- 42EC; 1xSSC, 50% formamide	65EC; 0.3xSSC
B	DNA:DNA	<50	T_B*; 1xSSC	T_B*; 1xSSC
C	DNA:RNA	$ 50	67EC; 1xSSC -or- 45EC; 1xSSC, 50% formamide	67EC; 0.3xSSC
D	DNA:RNA	<50	T_D*; 1xSSC	T_D*; 1xSSC
E	RNA:RNA	$ 50	70EC; 1xSSC -or- 50EC; 1xSSC, 50% formamide	70EC; 0.3xSSC
F	RNA:RNA	<50	T_F*; 1xSSC	T_f*; 1xSSC

G	DNA:DNA	$ 50	65EC; 4xSSC -or- 42EC; 4xSSC, 50% formamide	65EC; 1xSSC
H	DNA:DNA	<50	T_H*; 4xSSC	T_H*; 4xSSC
I	DNA:RNA	$ 50	67EC; 4xSSC -or- 45EC; 4xSSC, 50% formamide	67EC; 1xSSC
J	DNA:RNA	<50	T_J*; 4xSSC	T_J*; 4xSSC
K	RNA:RNA	$ 50	70EC; 4xSSC -or- 50EC; 4xSSC, 50% formamide	67EC; 1xSSC
L	RNA:RNA	<50	T_L*; 2xSSC	T_L*; 2xSSC
M	DNA:DNA	$ 50	50EC; 4xSSC -or- 40EC; 6xSSC, 50% formamide	50EC; 2xSSC
N	DNA:DNA	<50	T_N*; 6xSSC	T_N*; 6xSSC
O	DNA:RNA	$ 50	55EC; 4xSSC -or- 42EC; 6xSSC, 50% formamide	55EC; 2xSSC
P	DNA:RNA	<50	T_P*; 6xSSC	T_P*; 6xSSC
Q	RNA:RNA	$ 50	60EC; 4xSSC -or- 45EC; 6xSSC, 50% formamide	60EC; 2xSSC
R	RNA:RNA	<50	T_R*; 4xSSC	T_R*; 4xSSC

[I]: The hybrid length is that anticipated for the hybridized region(s) of the hybridizing polynucleotides. When hybridizing a polynucleotide to a target polynucleotide of unknown sequence, the hybrid length is assumed to be that of the hybridizing polynucleotide. When polynucleotides of known sequence are hybridized, the hybrid length can be determined by aligning the sequences of the polynucleotides and identifying the region or regions of optimal sequence complementarity.

[H]: SSPE (1xSSPE is 0.15M NaCl, 10mM NaH_2PO_4, and 1.25mM EDTA, pH 7.4) can be substituted for SSC (1xSSC is 0.15M NaCl and 15mM sodium citrate) in the hybridization and wash buffers; washes are performed for 15 minutes after hybridization is complete.

*T_B - T_R: The hybridization temperature for hybrids anticipated to be less than 50 base pairs in length should be 5-10EC less than the melting temperature (T_m) of the hybrid, where T_m is determined according to the following equations. For hybrids less than 18 base pairs in length, T_m(EC) = 2(# of A + T bases) + 4(# of G + C bases). For hybrids between 18 and 49 base pairs in length, T_m(EC) = 81.5 + 16.6($\log_{10}$[Na^+]) + 0.41(%G+C) - (600/N), where N is the number of bases in the hybrid, and [Na^+] is the concentration of sodium ions in the hybridization buffer ([Na^+] for 1xSSC = 0.165 M).

Additional examples of stringency conditions for polynucleotide hybridization are provided in Sambrook, J., E.F. Fritsch, and T. Maniatis, 1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, chapters 9 and 11, and Current Protocols in Molecular Biology, 1995, F.M. Ausubel et al., eds., John

Wiley & Sons, Inc., sections 2.10 and 6.3-6.4, incorporated herein by reference.

Preferably, each such hybridizing polynucleotide has a length that is at least 25%(more preferably at least 50%, and most preferably at least 75%) of the length of the polynucleotide of the present invention to which it hybridizes, and has at least 60% sequence identity (more preferably, at least 75% identity; most preferably at least 90% or 95% identity) with the polynucleotide of the present invention to which it hybridizes, where sequence identity is determined by comparing the sequences of the hybridizing polynucleotides when aligned so as to maximize overlap and identity while minimizing sequence gaps.

Expression of BBP1

The isolated polynucleotide of the invention may be operably linked to an expression control sequence such as the pMT2 or pED expression vectors disclosed in Kaufman et al., Nucleic Acids Res. 19, 4485-4490 (1991), in order to produce the protein recombinantly. Many suitable expression control sequences are known in the art. General methods of expressing recombinant proteins are also known and are exemplified in R. Kaufman, Methods in Enzymology 185, 537-566 (1990). As defined herein "operably linked" means that the isolated polynucleotide of the invention and an expression control sequence are situated within a vector or cell in such a way that the protein is expressed by a host cell which has been transformed (transfected) with the ligated polynucleotide/expression control sequence.

Expression Systems for BBP1

A number of types of cells may act as suitable host cells for expression of the protein. Mammalian host cells include, for example, monkey COS cells, Chinese Hamster Ovary (CHO) cells, human kidney 293 cells, human epidermal A431 cells, human Colo205 cells, 3T3 cells, CV-1 cells, other transformed primate cell lines, normal diploid cells, cell

strains derived from *in vitro* culture of primary tissue, primary explants, HeLa cells, mouse L cells, BHK, HL-60, U937, HaK or Jurkat cells.

Alternatively, it may be possible to produce the protein in lower eukaryotes such as yeast or in prokaryotes such as bacteria. Potentially suitable yeast strains include *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Kluyveromyces* strains, *Candida*, or any yeast strain capable of expressing heterologous proteins. Potentially suitable bacterial strains include *Escherichia coli*, *Bacillus subtilis*, *Salmonella typhimurium*, or any bacterial strain capable of expressing heterologous proteins. If the protein is made in yeast or bacteria, it may be necessary to modify the protein produced therein, for example by phosphorylation or glycosylation of the appropriate sites, in order to obtain the functional protein. Such covalent attachments may be accomplished using known chemical or enzymatic methods.

The protein may also be produced by operably linking the isolated polynucleotide of the invention to suitable control sequences in one or more insect expression vectors, and employing an insect expression system. Materials and methods for baculovirus/insect cell expression systems are commercially available in kit form from, e.g., Invitrogen, San Diego, California, U.S.A. (the MaxBac7 kit), and such methods are well known in the art, as described in Summers and Smith, Texas Agricultural Experiment Station Bulletin No. 1555 (1987), incorporated herein by reference. As used herein, an insect cell capable of expressing a polynucleotide of the present invention is "transformed."

The protein of the invention may be prepared by culturing transformed host cells under culture conditions suitable to express the recombinant protein. The resulting expressed protein may then be purified from such culture (i.e., from culture medium or cell extracts) using known purification processes, such as gel filtration and ion exchange chromatography. The purification of the protein may also include an affinity column containing agents which will bind to the

protein; one or more column steps over such affinity resins as concanavalin A-agarose, heparin-toyopearl7 or Cibacrom blue 3GA Sepharose7; one or more steps involving hydrophobic interaction chromatography using such resins as phenyl ether, butyl ether, or propyl ether; or immunoaffinity chromatography.

Alternatively, the protein of the invention may also be expressed in a form which will facilitate purification. For example, it may be expressed as a fusion protein, such as those of maltose binding protein (MBP), glutathione-S-transferase (GST) or thioredoxin (TRX). Kits for expression and purification of such fusion proteins are commercially available from New England BioLab (Beverly, MA), Pharmacia (Piscataway, NJ) and InVitrogen, respectively. The protein can also be tagged with an epitope and subsequently purified by using a specific antibody directed to such epitope. One such epitope ("Flag") is commercially available from Kodak (New Haven, CT).

Finally, one or more reverse-phase high performance liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media, e.g., silica gel having pendant methyl or other aliphatic groups, can be employed to further purify the protein. Some or all of the foregoing purification steps, in various combinations, can also be employed to provide a substantially homogeneous isolated recombinant protein. The protein thus purified is substantially free of other mammalian proteins and is defined in accordance with the present invention as an "isolated protein."

The protein of the invention may also be expressed as a product of transgenic animals, e.g., as a component of the milk of transgenic cows, goats, pigs, or sheep which are characterized by somatic or germ cells containing a nucleotide sequence encoding the protein.

The protein may also be produced by known conventional chemical synthesis. Methods for constructing the proteins of the present invention by synthetic means are known to those skilled in the art. The

synthetically-constructed protein sequences, by virtue of sharing primary, secondary or tertiary structural and/or conformational characteristics with proteins may possess biological properties in common therewith, including protein activity. Thus, they may be employed as biologically active or immunological substitutes for natural, purified proteins in screening of therapeutic compounds and in immunological processes for the development of antibodies.

The proteins provided herein also include proteins characterized by amino acid sequences similar to those of purified proteins but into which modification are naturally provided or deliberately engineered. For example, modifications in the peptide or DNA sequences can be made by those skilled in the art using known techniques. Modifications of interest in the protein sequences may include the alteration, substitution, replacement, insertion or deletion of a selected amino acid residue in the coding sequence. For example, one or more of the cysteine residues may be deleted or replaced with another amino acid to alter the conformation of the molecule. Techniques for such alteration, substitution, replacement, insertion or deletion are well known to those skilled in the art (see, e.g., USP No. 4,518,584). Preferably, such alteration, substitution, replacement, insertion or deletion retains the desired activity of the protein.

Other fragments and derivatives of the sequences of proteins which would be expected to retain protein activity in whole or in part and may thus be useful for screening or other immunological methodologies may also be easily made by those skilled in the art given the disclosures herein. Such modifications are believed to be encompassed by the present invention.

Yeast 2 Hybrid Assays

Y2H assays demonstrated that the association of BAP with the BBP1 fusion protein is specific. The association of BBP1 with BAP

suggests that BBP1 activity may have a defined role in the pathogenesis of Alzheimer's disease.

BBP1 sequences were compared to Genbank using the basic local alignment search tool (BLAST; Altschul et al., 1990). The BBP1 protein and translations of available expressed sequence tags were aligned, searched for conserved segments, and evaluated by the MoST (Tatusov et al., 1994) protein motif search algorithm. These analyses revealed a potential evolutionary relationship to the G protein-coupled receptor (GPCR) family. Specifically, these analyses indicated that BBP1 contains two potential transmembrane (tm) domains equivalent to tm domains 3 and 4 of G protein-coupled receptors. The intervening hydrophilic loop contains a well-characterized three amino acid motif, aspartate (D) or glutamate followed by arginine (R) and an aromatic residue (Y or F) (commonly referred to as the DRY sequence), that is conserved in almost all members of this receptor family and has been shown to serve as a molecular trigger for G protein activation (Acharya and Karnik, 1996).

Data from Y2H assays (see Figures 2-4) indicate that BBP1 represents a novel protein potentially containing a functional module shared with members of the G protein-coupled receptor superfamily. Specifically, it appears that BBP1 retains the critical DRF sequence (amino acids 199 to amino acids 201 of SEQ ID NO: 2), between two predicted tm domains, and may have the potential to couple to a G protein regulated signaling pathway.

APP has been shown to functionally associate with Gαo (Nishimoto et al., 1993; Yamatsuji et al., 1996) and BBP1 contains a structural motif known to be a Gα protein activating sequence in the related G protein-coupled receptors. Additionally, a hypothesis based on the predicted position and orientation of BBP1 tm domains suggests that the region of the protein that interacts with BAP would be topographically constrained to the same location as BAP in APP.

Y2H assay strains were engineered to evaluate the association of the BBP1 intracellular region with Gα proteins. The predicted intracellular sequences of BBP1 were expressed as a fusion protein and assayed for interactions with C-terminal regions of three Gα proteins. Protein segments used in these experiments are listed in Table 2, below. The BBP1 intracellular loop interacted with all three Gα proteins (Figure 3), supporting the premise that BBP1 may function as a modulator of G protein activity. These various Y2H assays suggest the intriguing model of a multiple protein complex minimally composed of the integral membrane proteins BBP1 and APP coupled to a heterotrimeric G protein.

Table 2. Plasmids used in yeast 2-hybrid assays

expression plasmid	protein		segment
	BAP		
pEK162		(human)	1 - 42
pEK240		(mouse)	1 - 42
	BBP1		
pEK196		(clone 14)	68 - 269
pEK198		(Δtm)	68 - 202
pEK219		(ΔC)	68 - 175
pEK216		(ΔN)	123 - 202
pOZ339		(intracellular)	185 - 217
	Gα		
pOZ345		(Gαs)	235 - 394
pOZ346		(Gαo)	161 - 302
pOZ348		(Gαi2)	213 - 355

Further analysis of BBP1 was obtained using Y2H assays. Two overlapping portions of the BBP1 sequences contained in the BBP1Δtm clone were amplified and cloned into the Y2H vector pACT2 (expression plasmids pEK216 and pEK219, Table 2 and corresponding proteins BBP1ΔN and BBP1ΔC, (Figure 4)). The ΔC construct lacked both tm domains; the ΔN construct encoded the first tm domain plus the preceding 52 amino acids. These fusion proteins were assayed with the BAP fusion protein and responses compared to those of strains expressing the larger BBP1Δtm protein. The BBP1ΔC protein induced a weak Y2H response (compare BBP1ΔC to vector, Figure 4), but the BBP1ΔN protein,

containing the first tm domain and adjacent amino-proximal sequences produced a response only slightly weaker than that observed with BBP1Δtm (Figure 4). These results suggest that a major determinant for the association with BAP is contained within the BBP1 region predicted to be topographically similar to BAP in the wild-type APP protein.

The Y2H system was utilized to demonstrate the selectivity and specificity of BBP1 binding to human BAP as compared to rodent BAP. There are three amino acid substitutions (G5R, F10Y and R13H) in the rodent BAP sequence compared to the human sequence. In a Y2H assay described in Example 6, the rodent peptide demonstrates reduced neurotoxicity and an absence of binding to human brain homogenates (Maggio et al., 1992). It was of interest, therefore, to evaluate the association of rodent BAP with BBP1 in the Y2H system. The sequence of human BAP in pEK162 was changed to encode the rodent peptide by oligonucleotide directed mutagenesis by PCR. The resultant plasmid, pEK240, is identical to the human BAP fusion protein expression plasmid utilized throughout this report except for the three codons producing the amino acid substitutions for the rodent peptide sequence. Interactions between BBP1 fusion protein and rodent and human BAP fusion proteins were compared by Y2H bioassay. Strains expressing BBP1 and the rodent BAP failed to produce a growth response (Figure 7). This finding supports the premise that BBP1 may serve as a specific mediator of the neurotoxic effects of BAP, and provides a mechanism to explain the reduced neurotoxicity of the rodent BAP. Importantly, these data also serve to illustrate the high degree of specificity of the BBP1/BAP interaction in the Y2H assays since the substitution of three amino acids was sufficient to completely abrogate the association (Figure 7).

Isolated BBP1 polypeptides

Proteins and protein fragments of the present invention include proteins with amino acid sequence lengths that are at least 25%(more preferably at least 50%, and most preferably at least 75%) of the length

of a disclosed protein and have at least 60% sequence identity (more preferably, at least 75% identity; most preferably at least 90% or 95% identity) with that disclosed protein, where sequence identity is determined by comparing the amino acid sequences of the proteins when aligned so as to maximize overlap and identity while minimizing sequence gaps. Also included in the present invention are proteins and protein fragments that contain a segment preferably comprising 8 or more (more preferably 20 or more, most preferably 30 or more) contiguous amino acids that shares at least 75% sequence identity (more preferably, at least 85% identity; most preferably at least 95% identity) with any such segment of any of the disclosed proteins.

Species homologues of the disclosed polynucleotides and proteins are also provided by the present invention. As used herein, a species homologue is a protein or polynucleotide with a different species of origin from that of a given protein or polynucleotide, but with significant sequence similarity to the given protein or polynucleotide. Preferably, polynucleotide species homologues have at least 60% sequence identity (more preferably, at least 75% identity; most preferably at least 90% identity) with the given polynucleotide, and protein species homologues have at least 30% sequence identity (more preferably, at least 45% identity; most preferably at least 60% identity) with the given protein, where sequence identity is determined by comparing the nucleotide sequences of the polynucleotides or the amino acid sequences of the proteins when aligned so as to maximize overlap and identity while minimizing sequence gaps. Species homologues may be isolated and identified by making suitable probes or primers from the sequences provided herein and screening a suitable nucleic acid source from the desired species. Preferably, species homologues are those isolated from mammalian species. Most preferably, species homologues are those isolated from certain mammalian species such as, for example, *Pan troglodytes*, *Gorilla gorilla*, *Pongo pygmaeus*, *Hylobates concolor*, *Macaca*

mulatta, Papio papio, Papio hamadryas, Cercopithecus aethiops, Cebus capucinus, Aotus trivirgatus, Sanguinus oedipus, Microcebus murinus, Mus musculus, Rattus norvegicus, Cricetulus griseus, Felis catus, Mustela vison, Canis familiaris, Oryctolagus cuniculus, Bos taurus, Ovis aries, Sus scrofa, and *Equus caballus*, for which genetic maps have been created allowing the identification of syntenic relationships between the genomic organization of genes in one species and the genomic organization of the related genes in another species (O'Brien and Seuanez, 1988, *Ann. Rev. Genet.* **22**: 323-351; O'Brien et al., 1993, *Nature Genetics* **3**:103-112; Johansson et al., 1995, *Genomics* **25**: 682-690; Lyons et al., 1997, *Nature Genetics* **15**: 47-56; O'Brien et al., 1997, *Trends in Genetics* **13(10)**: 393-399; Carver and Stubbs, 1997, *Genome Research* **7**:1123-1137; all of which are incorporated by reference herein).

The invention also encompasses allelic variants of the disclosed polynucleotides or proteins; that is, naturally-occurring alternative forms of the isolated polynucleotides which also encode proteins which are identical or have significantly similar sequences to those encoded by the disclosed polynucleotides. Preferably, allelic variants have at least 60% sequence identity (more preferably, at least 75% identity; most preferably at least 90% identity) with the given polynucleotide, where sequence identity is determined by comparing the nucleotide sequences of the polynucleotides when aligned so as to maximize overlap and identity while minimizing sequence gaps. Allelic variants may be isolated and identified by making suitable probes or primers from the sequences provided herein and screening a suitable nucleic acid source from individuals of the appropriate species.

The invention also includes polynucleotides with sequences complementary to those of the polynucleotides disclosed herein.

Applications

BBP1 proteins of the present invention can be used in a variety of applications routine to one of skill in the art based upon this disclosure.

Specifically the BBPs can be used as immunogens to raise antibodies which are specific to the cloned polypeptides. Various procedures known in the art may be used for the production of antibodies to BBP1 proteins. Such antibodies include, but are not limited to, polyclonal, monoclonal, chimeric, single chain, Fab fragments and an Fab expression library. For the production of antibodies, various host animals including, but not limited to rabbits, mice, and rats, are injected with a BBP. In one embodiment, the polypeptide or a fragment of the polypeptide capable of specific immunoactivity is conjugated to an immunogenic carrier. Adjuvants may also be administered in conjunction with the polypeptide to increase the immunologic response of the host animal. Examples of adjuvants which may be used include, but are not limited to, complete and incomplete Freund's, mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, and dinitrophenol.

Monoclonal antibodies to BBP1 proteins of the present invention can be prepared using any technique which provides for the production of antibodies by continuous cell line in culture. Such techniques are well known to those of skill in the art and include, but are not limited to, the hybridoma technology originally described by Kohler and Milstein (Nature 1975, 256,4202-497), the human B-cell hybridoma technique described by Kosbor et al. (Immunology Today 1983, 4, 72) and the EBV-hybridoma technique described by Cole et al. (Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp 77-96).

Antibodies immunoreactive to the polypeptides of the present invention can then be used to screen for the presence and subcellular distribution of similar polypeptides in biological samples. In addition, monoclonal antibodies specific to the BBP1 proteins of the present invention can be used as therapeutics.

The BBP1 proteins can also serve as antigens useful in solid phase assays measuring the presence of antibodies which immunoreact with the claimed peptides. Solid phase competition assays can be used to measure immunological quantities of clone 14-related antigen in biological samples. This determination is not only useful in facilitating the complete characterization of the cellular function or functions of the polypeptides of the present inventions, but can also be used to identify patients with abnormal amounts of these proteins.

BBP1 proteins of the present invention can also be used as capture reagents in affinity chromatography for the detection of BAP and BAP aggregates as markers for AD.

In addition, these BBP1s are useful as reagents in an assay to identify candidate molecules which effect the interaction of BAP and the cloned protein. Compounds that specifically block this association could be useful in the treatment or prevention of AD.

These BBP1s are also useful in acellular in vitro binding assays wherein alteration by a compound in the binding of these beta amyloid peptide associated proteins to BAP or BAP aggregates is determined. Acellular assays are extremely useful in screening sizable numbers of compounds since these assays are cost effective and easier to perform than assays employing living cells. Upon disclosure of the polypeptides of the present invention, the development of these assays would be routine to the skilled artisan. In such assays, either BBP1 or BAP is labeled. Such labels include, but are not limited to, radiolabels, antibodies, and fluorescent or ultraviolet tags. Binding of a BBP1 to BAP or BAP aggregates is first determined in the absence of any test compound. Compounds to be tested are then added to the assay to determine whether such compounds alter this interaction.

Examples

The present invention is further described by the following examples. The examples are provided solely to illustrate the invention by

reference to specific embodiments. These exemplifications, while illustrating certain specific aspects of the invention do not portray the limitations or circumscribe the scope of the invention.

Yeast two-hybrid system (hereinafter "Y2H"): Y2H expression plasmids were constructed in vectors pAS2 and pACT2 (described in WadeHarper et al., 1993) and pCUP (described in Ozenberger and Young, 1995). Yeast strain CY770 (Ozenberger and Young, 1995) served as the host for all Y2H assays.

Genetic screen: The polymerase chain reaction (PCR) method was used to amplify and modify sequences encoding BAP. Oligonucleotides #1 (5' - CC ATG GAT GCA GAA TTC CGA C) and #3 (5' - AAGCTTGTCGAC TTA CGC TATGAC AAC ACC GC) were used to amplify BAP using pCLL621, a modified human APP clone (Jacobsen et al., 1994), as template. The amplified DNA consists of codons 389 to 430 (which encodes BAP_{42}) of the APP precursor protein with the following modifications. The sense strand primer added a 5' *NcoI* restriction site in the same translational reading frame as the *NcoI* site in pAS2. The antisense strand primer added a stop codon and *HindIII* and *SalI* sites for cloning. The product from this amplification was ligated into the TA cloning system (Invitrogen Corp., Carlsbad, CA), and subsequently removed by digestion with *NcoI* and *SalI*. This fragment was cloned into pAS2 cleaved with *NcoI* plus *SalI*. The resultant plasmid, pEK162, was confirmed by DNA sequencing through the GAL4/BAP junction. The protein (BAP^{BD}; Figure 1) expressed from pEK162 comprised a fusion protein containing the DNA-binding domain of the yeast transcriptional activation protein Gal4 (lacking functional activation sequences) with the addition of the 42 amino acids of BAP to the carboxy-terminus. An expression plasmid was developed that mediates the expression of unmodified BAP_{42}. Oligo #2 (5' - AAGCTTAAG ATG GAT GCA GAA TTC CGA C) was paired with oligo #3 in a PCR as described above. The product of this amplification contains a 5' *HindIII* site and translation

initiation signals optimized for expression in *Saccharomyces cerevisiae*. Again, the DNA fragment was cloned into the TA system. It was then isolated on a *HindIII* fragment and cloned into pCUP cleaved with *HindIII*. The orientation of the BAP gene in the resultant plasmid, pEK149 (BAP; Figure 1), was confirmed by DNA sequencing. The BAP expression plasmids pEK149 (which used *URA3* as the selection marker) and pEK162 (which used *TRP1* as the selection marker) were transformed into the yeast host CY770 (Ozenberger and Young, 1995). The strain containing both plasmids was designated CY2091. A plasmid library consisting of cDNA fragments isolated from human fetal brain cloned into the yeast 2-hybrid expression vector pACT2 (which used *LEU2* as the selection marker) was purchased from Clontech Laboratories, Inc. (Palo Alto, CA). The library-derived protein is depicted in Figure1 as unknown^{AD}. This library was used to transform CY2091. The samples were spread on synthetic complete (SC) yeast growth medium lacking uracil, tryptophan, and leucine to select cells containing all three plasmids. The medium also lacked histidine and contained 3-amino-triazole, an inhibitor of the product of the yeast *HIS3* gene, at a concentration of 25 mM. 3-Amino-triazole was utilized to reduce activity from low-level constitutive expression of the *HIS3* reporter gene. Plates were incubated at 30°C for 12 days. Twenty-four colonies exhibiting increased histidine prototrophy were isolated. Transformation controls indicated that the screen assayed 10^6 individual clones. A PCR approach was utilized to quickly determine the content of positive clones. Total DNA was isolated from each positive strain by standard methods. This material was used as template for PCRs using oligos #4 (5' - TTTAATACCA CTACAATGGA T) plus #5 (5' - TTTTCAGTAT CTACGATTCA T) which flank the cloning region of the library vector pACT2. DNA fragments were ligated into the TA system and examined by DNA sequencing. The library plasmid contained in clone #14 (as described above) was isolated by shuttle into *E. coli*. The

nucleotide sequence of the human cDNA sequences was determined, confirming the sequence of the initial PCR product.

Bioassays: Strains were grown overnight in 2ml SC medium lacking leucine and tryptophan to a density of approximately 7 x 10^7 cells per ml. Cells were counted and 10-fold serial dilutions made from 10^4 to 10^8 cells per ml in sterile water. These samples were spotted in 5 µl aliquots on SC medium lacking leucine, tryptophan and histidine and containing 25 mM 3-amino-triazole. Plates were incubated at 30°C for 2 to 3 days. Positive protein/protein interactions were identified by increased prototrophic growth compared to control strains expressing the Gal4 DNA-binding domain fusion protein plus an irrelevant transcriptional activation domain fusion protein (or simply containing the pACT vector without inserted sequences). These control strains were indicated in the Figures described above as the label 'vector'. This assay method was highly reproducible and provided for the detection of subtle inductions of growth mediated by the specific interaction between target proteins. The original BBP1 clone, designated pEK196 and deposited as ATCC 98399; is referred herein as clone 14), was used as a PCR template to truncate the protein product to express BBP1Δtm. Sense primer #6 (5'-TTTAATACCA CTACAATGGA T) annealed to GAL4 sequences in pACT2. The antisense primer #7 (5'-CTCGAG TTA AAA TCG ATC TGC TCC CAA CC) incorporated a 3' stop codon and *Xhol* site immediately 3' to the sequences encoding the DRF motif of BBP1. The PCR product was ligated into the TA cloning vector and subsequently digested with *Eco*RI + *Xhol* and cloned into pACT2. The hybrid product expressed from this plasmid (pEK198) was denoted BBP1Δtm. Similarly, primer #7 was paired with primer #8 (5'-GAATT CCA AAA ATA AAT GAC GCT ACG) to engineer the BBP1ΔN expression plasmid pEK216. Again, the PCR product was ligated into the TA system and the resultant plasmid digested with *Eco*RI + *Xhol* with the BBP1 fragment (codons 123-202) finally ligated into pACT2 digested with the same enzymes. BBP1ΔC was

made by using the pACT2-specific oligo #6 with antisense oligo #9 (5'-CTCGAG TCA AGA TAT GGG CTT GAA AAA AC). After TA cloning, isolation of the *Eco*RI-*Xhol* fragment and cloning into pACT2, the resultant plasmid, pEK219, expressed BBP1 from residue 68 to 175. Sequences encoding the BBP1 intracellular loop were amplified using oligonucleotides #10 (5'-CCTTCC ATG GAA GTG GCA GTC GCA TTG TCT) plus #11 (5'-AACACTCGAG TCA AAA CCC TAC AGT GCA AAA C). This product, containing BBP1 codons 185 to 217, was digested with *Ncol* + *Xhol* and cloned into pAS2 cleaved with *Ncol* + *Sall* to generate pOZ339.

Construction of all Gα protein expression plasmids utilized the *Bam*HI site near the center of each rat cDNA sequence (Kang et al., 1990) as the site of fusion in pACT2. Sense primers annealed to sequences 5' of the *Bam*HI site; antisense primers annealed to sequences 3' of the stop codon and included a SalI restriction site. Primers were: Gαo, sense (#17) = 5'-GTGGATCCAC TGCTTCGAGG AT, antisense (#18) = 5'-GTCGACGGTT GCTATACAGG ACAAGAGG; Gas, sense (#19) = 5'-GTGGATCCAG TGCTTCAATG AT, antisense (#20) = 5'-GTCGACTAAA TTTGGGCGTT CCCTTCTT; Gai2, sense (#21) = 5'-GTGGATCCAC TGCTTTGAGG GT, antisense (#22) = 5'-GTCGACGGTC TTCTTGCCCC CATCTTCC. PCR products were cloned into the TA vector. Gα sequences were isolated as *Bam*HI-*Sall* fragments and cloned into pACT2 digested with *Bam*HI + *Sall*. See Table 2 for plasmid designations. Finally, oligonucleotide #23 was synthesized for the conversion of human BAP to the rodent sequence. This primer has the sequence 5'-ATATGGCCATG GAT GCA GAA TTC GGA CAT GAC TCA GGA TTT GAA GTT CGT. The triplets represent the first 13 codons of BAP; the three nucleotides that were changed to produce the rodent sequence are underlined. Oligo #23 was paired with #24 (5'-TGACCTACAG GAAAGAGTTA) which anneals to a region of the Y2H vectors that is 3' of the cloning site in a PCR using pEK162 as the template. The product was cleaved with *Ncol*+*Sall* and ligated into pAS2 to produce pEK240.

The nucleotide sequence of the segment encoding rodent BAP was confirmed.

Genomic cloning; RACE (rapid amplification of cDNA ends): A human genomic lambda library (Stratagene), corresponding to [a]2.0 X 10^6 pfus, was screened with randomly-primed *Eco*RI/ClaI fragment probe corresponding to nucleotides 187-600 (Figure 2). The probe was labeled with [^{32}P]-CTP using the ^{T7}QuickPrimer Kit according to the manufacturer's (Pharmacia) protocol. Filters were hybridized under high stringency: 40_C in 50% formamide, 0.12M $NaHPO_4$, 0.25M NaCl, 7% SDS and 25mg/ml sonicated salmon sperm DNA and washed at 65_C in 0.1X SSC containing 0.1% sodium dodecyl sulfate and exposed to Kodak BioMax MS film. Lambda phage clones hybridizing to the probe were plaque purified by successive plating and rescreening. Ten positive clones were purified and subjected to further analysis by hybridization to a 45 nt oligonucleotide probe directed to the most 5′ sequences known from the original cDNA clone. This oligonucleotide was the reverse complement of nucleotides. 157-201 (Figure 2) and has the sequence 5′-CCAGGCGGCC GCCATCTTGG AGACCGACAC TTTCTCGCCA CTTCC. Lambda phage DNA was isolated by standard molecular biology techniques and subjected to direct sequencing using fluorescent dideoxy cycle sequencing on an ABI 373 sequencer.

RACE: First strand DNA synthesis was performed using the r*Tth* thermal-stable polymerase system (Perkin Elmer). The following reagents were combined in a 1.5 mL tube to give a 10 microliter volume: 1X reverse transcription buffer, 1 mM $MnCl_2$, 1.6 mM dNTP mix, 2.5U rTth polymerase, 100ng human hippocampus poly A^+ RNA (Clontech), 10mM oligonucleotide (nt 429-452, Figure 2; 5′-GTTATGTTGG GTGCTGGAAA ACAG). The reaction was incubated at 70°C for 15 minutes and immediately placed on ice. The Marathon cDNA synthesis kit (Clontech) was used for second strand cDNA generation. The entire 10μl from the first strand reaction was combined with the following reagents: 1X

second strand buffer, 0.8 mM dNTP mix, 4X second strand cocktail (*E.coli* DNA polymerase I, *E.coli* DNA ligase, *E.coli* RNaseH), and dH_2O up to a volume of 80µl. The tube was incubated at 16–C for 1.5 hours after which time T4 DNA polymerase (10U) was added and incubated for an additional 45 minutes at 16–C. To terminate the reaction, 4µl of 20X EDTA/glycogen (0.2M EDTA/2mg/ml glycogen) was added to the reaction mixes followed by a phenol/chloroform/isoamyl alcohol extraction to remove enzymes and other impurities. The DNA was precipitated by adding 0.1X volume 3M Na acetate pH 5.2 and 2.5X volume reagent grade EtOH and place at -70–C. The DNA was washed once with 70% EtOH, dried down and resuspended in 10µl dH_2O. Half of the DNA was used for Marathon adaptor ligation to be used in subsequent RACE PCR reactions following the Clontech protocol as follows: 5µl cDNA was added to 2µl (10mM) Marathon (5'- CTAATACGAC TCACTATAGG GCTCGAGCGG CCGCCCGGGC AGGT), 1X DNA ligation buffer and 1µl (1U) T4 DNA ligase. The reaction mix was incubated overnight at 16–C. The mix was diluted 1:50 for initial RACE reaction and combined in a 0.2mL PCR tube with the following: 40µl dH_2O, 1µl 10X Klentaq DNA polymerase (Clontech), 1µl (10mM) AP1 primer (5'-CCATCCTAAT ACGACTCACT ATAGGGC), 1µl (10mM) BBP1-specific primer (corresponding to nts. 187-209, Figure 2; 5'-CCAGACGGCCA GGCGGCCGCC AT), 5µl 10X Klentaq polymerase buffer, 1µl 10mM dNTP mix, 1µl of diluted cDNA from above reaction. The following cycling conditions were performed using a Perking Elmer GeneAmp PCR system 2400 thermocycler: Denaturing cycle 94–C for 1 minute followed by 5 cycles of 30" at 94°C, 3' at 72°C, 5 cycles of 30" at 94°C, 3' at 70°C, followed by 25 cycles of 30" at 94°C, 3' at 68°C, with a final extension 7' at 72°C. This was followed by a nested RACE PCR reaction as follows: 40µl dH_2O, 1µl (1U) 10X AmplitaqGold DNA polymerase (Perkin Elmer), 1µl (10mM) AP2 primer (5'-ACTCACTATA GGGCTCGAGC GGC),

1μl (10mM) BBP1-specific primer (corresponding to nts. 172-194, Figure 2; 5'-GCCGCCATCT TGGAGACCGA CAC), 5μl 10X Amplitaq polymerase buffer, 1μl 10mM dNTP mix, 1μl of primary RACE product. The PCR cycling conditions were an initial denaturing cycle of 9' at 94°C, 25 cycles of 30" at 94°C, 30" at 68°C, 2' at 72°C, followed by a 72°C extension for 7'. The PCR product was run on a 1% agarose gel in 1XTBE buffer. The resulting 350 base pairs product was gel purified and directly cloned using the TA Cloning Kit (Invitrogen). Ligation mixes were transformed into OneShot Cells (Invitrogen) and plated on LB-ampicillin (100μg/ml) agar plates containing X-gal. Mini-prep DNA was obtained and examined by fluorescent dideoxy cycle sequencing on an ABI 373 sequencer.

Northern analyses. Human multiple tissue and multiple brain tissue mRNA Northern blots were obtained from Clontech (Palo Alto, CA). BBP1 sequences extending from the original fusion junction to the poly-A region were isolated on an *Eco*RI fragment from a TA clone derived from pEK196. β-actin DNA was provided by the manufacturer. Radiolabelled probes were produced from these DNAs using a random priming method to incorporate ^{32}P-dCTP (Pharmacia Biotech, Piscataway, NJ). Hybridizations were performed per manufacturer's (Clontech) instructions in Express Hyb Solution at 68°C. Blots were washed in 2x SSC (1X SSC is 0.15 M sodium chloride, 0.015 M sodium citrate), 0.05% SDS at room temperature, followed by two washes in 0.1 x SSC, 0.1% SDS at 50°C. Hybridization signals were visualized by exposure to Kodak BioMax film.

In situ hybridization. DNA templates for riboprobe synthesis were prepared by PCR using a plasmid clone containing the full length human BBP cDNA. A single riboprobe targeted to the 3' UTR of the cDNA was used. The probe sequences were checked versus the GenBank database to ensure that they only recognize the appropriate targets among all deposited sequences. To generate riboprobes for BBP1, a pair of oligonucleotide primers was designed to amplify a 275 base pairs region

from the 3' UTR of the BBP1 cDNA and, in addition, add the promoter sequences for T7 (sense) and T3 (antisense) polymerase. These primers contained the following sequences: 5'-TAATACGACT CACTATAGGG TTAGAAGAAA CAGATTTGAG (forward); 5'-ATTAACCCTC ACTAAAGGGA CAAGTGGCAA CTTGCCTTTG (reverse). PCR products were gel purified on 1.5% low-melt agarose gels, and bands containing the products were excised, phenol and phenol-chloroform extracted, and ethanol precipitated. Pellet were dried and resuspended in 1X TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 7.4). The APP riboprobe template consisted of a *DdeI-XhoI* fragment from the protein coding region, as described by Jacobsen et al. (1991). Fifty ng of DNA template was used for transcription reactions using (^{35}S)-CTP (New England Nuclear, Boston, MA) and the Riboprobe Gemini™ System (Promega, Madison, WI).

In situ hybridization histochemistry using sections of postmortem human hippocampus were performed as described previously (Rhodes, 1996). Sections were cut at 10 μm on a Hacker-Brights cryostat and thaw-mounted onto chilled (-20°C) slides coated with Vectabond reagent (Vector Labs, Burlingame, CA). All solutions were prepared in dH_2O treated with 0.1% (v/v) diethylpyrocarbonate and autoclaved. Sections were fixed by immersion in 4% paraformaldehyde in PBS (pH 7.4) then immersed sequentially in 2xSSC, dH_2O, and 0.1M triethanolamine, pH 8.0. The sections were then acetylated by immersion in 0.1M triethanolamine containing 0.25% (v/v) acetic anhydride, washed in 0.2xSSC, dehydrated in 50, 70 and 90% ethanol, and rapidly dried. One ml of prehybridization solution containing 0.9M NaCl, 1mM EDTA, 5x Denhardt's, 0.25 mg/ml single-stranded herring sperm DNA (GIBCO/BRL, Gaithersberg, MD), 50% deionized formamide (EM Sciences, Gibbstown, NJ) in 10mM Tris, (pH 7.6), was pipetted onto each slide, and the slides incubated for 3 hrs. at 50°C in a humidified box. The sections were then dehydrated by immersion in 50, 70, and 90% ethanol and air dried. Labeled riboprobes were added at a final concentration of 50,000 cpm/μl

to hybridization solution containing 0.9M NaCl, 1mM EDTA, 1x Denhardt's, 0.1 mg/ml yeast tRNA, 0.1 mg/ml single-stranded salmon sperm DNA, dextran sulfate (10%), 0.08% BSA, 10mM DTT (Boehringer Mannheim, Indianapolis, IN), and 50% deionized formamide in 10mM Tris (pH 7.6). The probes were then denatured at 95°C (1 min), placed on ice (5 min), and pipetted onto the sections and allowed to hybridize overnight at 55°C in a humidified chamber. The sections were subsequently washed 1 x 45 min at 37°C in 2xSSC containing 10mM DTT, followed by 1 x 30 min at 37°C in 1xSSC containing 50% formamide, and 1 x 30 min at 37°C in 2xSSC. Single stranded and non-specifically hybridized riboprobe was digested by immersion in 10mM Tris pH 8.0 containing bovine pancreas RNAse A (Boehringer Mannheim; 40 mg/ml), 0.5M NaCl, and 1mM EDTA. The sections were washed in 2XSSC for 1 hr at 60°C, followed by 0.1XSSC containing 0.5% (w/v) sodium thiosulfate for 2 hrs. at 60°C. The sections were then dehydrated in 50, 70, 90% ethanol containing 0.3M ammonium acetate, and dried. The slides were loaded into X-ray cassettes and opposed to Hyperfilm b-Max (Amersham) for 14-30 days. Once a satisfactory exposure was obtained, the slides were coated with nuclear-track emulsion (NTB-2; Kodak) and exposed for 7-21 days at 4°C. The emulsion autoradiograms were developed and fixed according to the manufacturer's instructions, and the underlying tissue sections were stained with hematoxylin. To assess nonspecific labeling, a control probe was generated from a template provided in the Riboprobe Gemini™ System kit (Promega). This vector was linearized using ScaI and transcribed using T3 polymerase. The resulting transcription reaction generates two products, a 250 base and a 1,525 base riboprobe, containing only vector sequence. This control probe mixture was labeled as described above and added to the hybridization solution at a final concentration of 50,000 cpm/μl. No specific hybridization was observed in control sections, i.e., these sections gave a very weak uniform

hybridization signal that did not follow neuroanatomical landmarks (data not shown).

Example 1: Cloning and Isolation BAP-binding protein (BBP1).

A yeast 2-hybrid (Y2H) genetic screen was developed to identify proteins which interact with human BAP_{42}, a 42 amino acid proteolytic fragment of APP which is considered to potentially be the more toxic aggregated form of BAP. BAP_{42} was expressed fused to the yeast Gal4 DNA-binding domain and was also expressed as free peptide (Figure 1). This strain was transformed with a human fetal brain cDNA Y2H library. A single clone, designated clone14 defined above, from approximately 10^6 independent transformants, produced consistent reporter gene activation and contained a substantial open reading frame continuous with that of the GAL4 domain. The cDNA insert comprised 984 base pairs, terminating in a poly-A tract. This sequence encoded 201 amino acids (SEQ ID NO: 2; amino acid residues 68 to 269) with two regions of sufficient length and hydrophobicity to transverse a cellular membrane.

The library-derived plasmid was isolated from clone 14 and used to reconstruct Y2H assay strains. Examination of these strains demonstrated that the BAP fusion protein specifically interacted with the clone 14 protein, although the response was weak. Since protein domains of strong hydrophobicity, such as transmembrane regions, inhibit Y2H responses (Ozenberger, unpublished data), clone 14 insert was truncated (hereinafter BBP1Δtm) to remove the region of strongest hydrophobicity and retested for interactions with BAP. A much more robust Y2H response was observed with BBP1Δtm (Figure 2), supporting the notion that the deleted sequences encode a potential transmembrane ("tm") anchor. The nucleotide sequence of Clone 14 was searched against GenBank; the BAP binding protein (BBP1) thus identified appeared to be novel.

Example 2: Isolation and confirmation of the 5' terminus of BBP1.

The BBP1 cDNA sequences contained in clone 14 described in Example 1, above, lacked the 5' end of the protein coding region as no potential initiating methionine codon was present. Multiple attempts at conventional 5' RACE (rapid amplification of cDNA ends) utilizing a standard reverse-transcriptase only resulted in the addition of 27 nucleotides. These sequences included an ATG, but no upstream stop codon in the same translational reading frame to provide confidence that this was the initiating codon. A genomic cloning approach was initiated to isolate the 5' terminus of the BBP1 gene.

Hybridization of a human genomic lambda library with a randomly-primed probe corresponding to 400 base pairs (bps) of the 5' sequence of clone 14 resulted in identification of 10 positive clones. Further characterization of these clones using a 45-base oligonucleotide probe directed to the most upstream BBP1 sequence of clone 14 (and corresponding to the 5' upstream sequence of the 400 base pairs probe revealed that 6 of the 10 clones included the terminal 5' sequences contained within those previously identified. It was determined that the other 4 lambda clones represented other exons which were contained within the original 400 base pairs randomly-primed cDNA-derived probe (data not shown). Direct cycle sequencing of lambda phage DNA from representative clones corresponding to the 5' end of BBP1 revealed ^{a}500 nucleotides upstream and overlapping with the sequence known for clone 14. This additional sequence potentially encodes 62 additional amino acids upstream of the previously characterized MET before arriving at a MET preceded by an in-frame stop codon. Although there exist two MET residues downstream from the furthest upstream MET, by standard convention we have tentatively defined the sequence of the amino terminus of the human BBP1 gene to include the first 5' MET which follows an in-frame stop codon. The entire coding region and deduced protein sequence is shown in SEQ ID NOS:1 and 2. A plasmid (denoted

BBP1-fl) containing this amino acid sequence has been deposited in the American Type Culture Collection having accession number 98617).

Since the 5' coding sequences were derived from a genomic library, there existed the possibility that this region contained introns. This potentiality was investigated by two methods. First, a forward primer directed to the region of the 5' MET and a reverse primer within the original clone 14 were utilized to amplify sequences from brain cDNA as well as from genomic DNA. Products of identical size were generated from both samples, indicating the absence of introns within this region and confirming the linkage of the upstream sequence with the original sequence. Secondly, cDNA sequences were isolated in modified 5' RACE experiments (see Materials and Methods, above) that were identical to those obtained from the genomic clone. These findings confirmed the upstream sequences (both from genomic and cDNA sources) and the lack of introns in this region.

Example 3: Characterization of BBP1.

BBP1 sequences were compared to Genbank using the basic local alignment search tool (BLAST; Altschul et al., 1990). Two *Caenorhabditis elegans* and one *Drosophila melanogaster* genomic sequence and a large number of human, mouse and other mammalian expressed sequence tags were identified. However, no complete cDNA sequences were available nor were any functional data attributed to the gene. The BBP1 protein and translations of available expressed sequence tags were aligned, searched for conserved segments, and evaluated by the MoST (Tatusov et al., 1994) protein motif search algorithm. These analyses revealed a potential evolutionary relationship to the G protein-coupled receptor family. Specifically, these analyses indicated that BBP1 contains two potential transmembrane (tm) domains equivalent to tm domains 3 and 4 of G protein-coupled receptors. The intervening hydrophilic loop contains a well-characterized three amino acid motif, aspartate (D) or glutamate followed by arginine (R) and an aromatic residue (Y or F) (commonly

referred to as the DRY sequence), that is conserved in almost all members of this receptor family and has been shown to serve as a molecular trigger for G protein activation (Acharya and Karnik, 1996). These data indicate that BBP1 represents a novel protein potentially containing a functional module shared with members of the G protein-coupled receptor superfamily. Specifically, it appears that BBP1 retains the critical DRF sequence between two predicted tm domains, so may have the potential to couple to a G protein regulated signaling pathway.

Structural analysis of BBP1 indicated it contained a structural motif known to be a Gα protein activating sequence in the related G protein-coupled receptors. Y2H assays demonstrating the interaction of BBP1 with various members of the G protein coupled receptors are illustrated in Figure 3. Based on structural predictions, BBP1 is depicted as transversing a membrane twice with both termini in the lumenal compartment. Other orientations cannot be entirely ruled out. The potential protein interactions described above were investigated in Y2H assays. Two overlapping portions of the BBP1 sequences contained in the BBP1Δtm clone were amplified and cloned into the Y2H vector pACT2 (expression plasmids pEK216 and pEK219, Table 2 and corresponding proteins BBP1ΔN and BBP1ΔC, Figure 4). The ΔC construct is lacking both tm domains; the ΔN construct encodes the first tm domain plus the preceding 52 amino acids. These fusion proteins were assayed with the BAP fusion protein and responses compared to those of strains expressing the larger BBP1Δtm protein. These results suggest that a major determinant for the association with BAP is contained within the BBP1 region predicted to be topographically similar to BAP in the wild-type APP protein.

Example 4: <u>Tissue distribution of human BBP1 expression.</u>

Expression of BBP1 mRNA was evaluated as an initial step in elucidating the activity of the gene and its product. A major transcript of 1.25 kb was observed in all tissues (Figure 5A). There was a high level

of expression in heart. Whole brain exhibited an intermediate level of expression. Samples derived from separate brain regions all exhibited BBP1 expression (Figure 5B). Interestingly, limbic regions contained relatively greater amounts of BBP1 mRNA. These are the regions of the brain where BAP aggregation and associated neurotoxicity initially occur. Analysis of in situ hybridization autoradiograms obtained using a BBP1-specific riboprobe indicated that in human hippocampus and entorhinal cortex, BBP1 mRNA is expressed in medium to large cells in a pattern consistent with expression in neurons as opposed to glial cells (Figure 6). Moreover, BBP1 mRNA is expressed in virtually all hippocampal and entorhinal neurons, i.e., there do not appear to be any real or laminar differences in the intensity of the hybridization signal. Interestingly, the pattern of BBP1 expression was strikingly similar to the pattern observed using a riboprobe directed against mRNA for the amyloid precursor protein APP (Figure. 6). In summary, BBP1 mRNA was observed in all tissues and all brain regions examined. In situ analysis of BBP1 mRNA expression also revealed extensive expression in the hippocampus region.

Example 5: <u>Cell line distribution of BBP1 expression.</u>

BBP1 expression was also investigated in numerous cell lines and data were extracted from dbEST, the collection of expressed sequence tags from the National Center for Biotechnology Information. Reverse-transcription polymerase chain reaction (RT-PCR) methods were utilized to qualitatively assess BBP1 mRNA expression in cell lines commonly utilized for recombinant protein expression as well as a variety of cancer cell lines. BBP1 was observed in hamster CHO and human HEK293 cells. Signals were observed in the embryonic stem cell line Ntera-2 and neuroblastoma lines IMR32 and SK-N-SH. BBP1 expression was observed in cancer cell lines representing the following tissue origins: colon (Cx-1, Colo205, MIP101, SW948, CaCo, SW620, LS174T), ovarian (A2780S, A2780DDP), breast (MCF-7, SKBr-3, T47-D, B7474), lung (Lx-1, A5439), melanoma (Lox, Skmel30), leukemia (HL60, CEM), prostate (LNCAP,

Du145, PC-3). A Northern blot probing mRNA isolated from the following cancer cell lines demonstrated BBP1 expression in all samples: promyelocytic leukemia (HL-60), carcinoma (HeLa S3), chronic myelogenous leukemia (K-562), lymphoblastic leukemia (MOLT-4), Burkitt's lymphoma (Raji), colorectal adenocarcinoma (SW480), lung carcinoma (A549), and melanoma (G361).

Example 6: Selective interaction of BBP1 with human BAP versus rodent BAP

There are three amino acid substitutions (G5R, F10Y and R13H) in the rodent BAP sequence compared to the human sequence. The rodent peptide demonstrated reduced neurotoxicity and an absence of binding to human brain homogenates (Maggio et al., 1992). It was of interest, therefore, to evaluate the association of rodent BAP with BBP1 in the Y2H system. The sequence of human BAP in pEK162 was changed to encode the rodent peptide by oligonucleotide directed mutagenesis by PCR, described above. The resultant plasmid, pEK240, was identical to the human BAP fusion protein expression plasmid utilized throughout the present invention except for the three codons producing the amino acid substitutions for the rodent peptide sequence. Interactions between BBP1 fusion protein and rodent and human BAP fusion proteins were compared by Y2H bioassay. Strains expressing BBP1 and the rodent BAP failed to produce a growth response (Figure 7). This finding supports the premise that BBP1 may serve as a specific mediator of the neurotoxic effects of BAP, and provides a mechanism to explain the reduced neurotoxicity of the rodent BAP. Importantly, these data also serve to illustrate the high degree of specificity of the BBP1/BAP interaction in the Y2H assays since the substitution of three amino acids was sufficient to completely abrogate the association (Figure 7).

It is clear that the invention may be practiced otherwise than as particularly described in the foregoing description and examples. Numerous modifications and variations of the present invention are possible in light of the above teachings and therefore are within the scope of the appended claims.

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